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The calcitonin receptor is the main mediator of LAAMA's body weight lowering effects in male mice

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ABSTRACT

The anorectic action of the pancreatic hormone amylin is mainly mediated through the area postrema (AP). Amylin activates AP neurons using a heterodimeric receptor (AMY) composed of the calcitonin receptor (CTR) and the receptor activity modifying protein (RAMP 1, 2 or 3). The aim of the following experiments is to test the effects of the long acting amylin analogue (LAAMA) in RAMP1/3 knock-out (KO) male mice and in neuronal CTR KO Nestin-Cre^{CTR} male mice. In vitro, LAAMA exerted an equipotent effect on CTR and AMYs that was maintained across species. Following one week of 45% high fat diet, WT, RAMP1/3 KO and Nestin-Cre^{CTR} mice were injected daily for one week with vehicle or LAAMA. LAAMA decreased body weight gain in WT and in RAMP1/3 KO mice suggesting that RAMP1/3 are not necessary for LAAMA-induced effects. However, LAAMA was not able to produce any body lowering and anorectic effects in Nestin-Cre^{CTR} mice. This was accompanied by the absence of any c-Fos signal in the AP opposite to WT control mice. Together, these results suggest that LAAMA's effects are mainly mediated through CTR rather than specific AMY. The study of LAAMA or any amylin receptor agonist in different receptor KO mouse models helps disentangle the underlying mechanisms used by these molecules.

1. Introduction

The pancreatic peptide hormone amylin is produced in response to food intake and increased blood glucose levels (Hay et al., 2015; Le Foll and Lutz, 2020). Amylin primarily acts on the area postrema (AP) and its main effects are the induction of meal termination, the reduction of food intake, gastric emptying and body weight (Boyle et al., 2018; Lutz, 2009). Amylin and its receptor agonist, salmon calcitonin, are also known to increase energy expenditure in rodents (Coester et al., 2020; Wielinga et al., 2007, 2010; Zhang et al., 2011).

The amylin receptor (AMY) consists of the calcitonin receptor (CTR) core and one of the three receptor activity modifying proteins (RAMP1, RAMP2, RAMP3), which form AMY 1–3 respectively. Although CTR alone can bind amylin, RAMP increases CTR's affinity towards it (Christopoulos et al., 1999; Hay et al., 2015; McLatchie et al., 1998). Most CTR expressing cells in the AP express more than one RAMP subtypes (Liberini et al., 2016), and AMY1 and AMY3 seem to be the main mediators of amylin's effects (Bailey et al., 2012; Coester et al., 2020). The role of RAMP2 and AMY2 in the mediation of food intake and metabolism is more elusive, since specific antagonists are not available and since the RAMP2 whole body knockout is lethal (Kadmiel et al., 2011). RAMP and CTR have also been shown to be expressed in the medio-basal hypothalamus, lateral hypothalamus as well as in ventral tegmental area (Christopoulos et al., 1995, 1999; Coester et al., 2020; Sexton et al., 1986, 1994) but co-expression of the various AMY components in single cells in these areas has not been tested.

Amylin activates several intracellular pathways, namely it increases intracellular cyclic GMP and ERK phosphorylation which is associated with amylin's food intake reducing effects (Potes et al., 2012; Riediger et al., 2001, 2004). In vitro studies show that amylin binding increases cAMP, intracellular Ca^{2+} and beta-arrestin expression (Bower and Hay, 2016; Hay et al., 2005). Amylin also increases the expression of the neuronal activation marker c-Fos in its target cells.

RAMPs also bind to another member of calcitonin family receptors, namely the calcitonin receptor-like receptor (CLR). CLR and RAMP1 form calcitonin gene-related peptide (CGRP) receptor (CGRPR, CGRPR1) but AMY1 can also mediate the effects of CGRP (Bailey et al., 2012) (possibly representing the CGRPR2). Association of CLR with

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either RAMP2 or RAMP3 results in formation of adrenomedullin receptor (ADMR) (Poyner et al., 2002; Qi et al., 2008). These structural and functional similarities, between calcitonin family members, complicates studying AMY's physiology and pharmacology (Barwell et al., 2012). But, regardless of complex pharmacology, amylin's food intake and body weight reducing effects make it an attractive target for an anti-obesity and anti-diabetic drug development, with the amylin analogue pramlintide being already on the market (Hoogwerf et al., 2008; Ravussin et al., 2009).

In this study, we investigated the *in vitro* pharmacology of the long-acting amylin analogue (LAAMA) and its *in vivo* effects in RAMP1/3 knockout (KO) and neuronal CTR KO mice with the goal to shed light on the role of RAMPs and CTR in mediating the effects of LAAMA.

2. Materials and methods

2.1. Pharmacokinetic (PK) analysis of LAAMA in plasma

For PK analysis three lean RJHAN:WI male rats were dosed i.v. or s.c. with a single dose of 10 or 20 nmol/kg, respectively. Blood samples were collected at different timepoints post dosing in Microvette® tubes 0.50 ml K3EDTA (Sarstedt). The Microvette® tubes were ice-chilled prior to sampling. After collection, the blood was gently mixed by inverting the tube several times and stored upright on ice until centrifugation. Blood samples were centrifuged for 5 min at 8.300×*g* at 4 °C. After centrifugation, plasma was aliquoted and stored below −70 °C until further analysis. Plasma concentrations of LAAMA were analyzed using a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method with a calibration range of 1–1000 nM on a QTRAP 6500+ (Sciex, Framingham, MA, USA). Samples were pretreated with ethanol for protein precipitation before the analysis. All procedures were approved by the German Veterinary Office under the license n° 14-009-G.

2.2. Functional cAMP assay with stable cell lines

The *in vitro* functional cAMP assay was used to measure accumulation of cAMP after receptor activation in cells stably expressing human CTR (1321N1 CALCR Gs Cell Line cAMP Hunter™, DiscoverX), human CGRPR (1321N1 CALCRL RAMP1 Gs Cell Line cAMP Hunter™, DiscoverX) or human ADMR (1321N1 CALCRL RAMP3 Gs Cell Line cAMP Hunter™, DiscoverX). Intracellular cAMP increase was measured using the AlphaScreen™ cAMP Functional Assay Kit (Perkin Elmer) according to the manufacturer's protocol. Briefly, 5000 cells in 50 µl culture medium were seeded per well in 384-well plates and incubated over night at 37 °C and 5% CO_2. On the day of assay, test compounds were reconstituted in DMSO to a 1 mM stock solution and then diluted in assay buffer containing 1% DMSO. The assay was started by substitution of the culture medium with 10 µl assay buffer containing test compounds. Cells were stimulated with the compounds for 45 min at RT. The stimulation was stopped by adding 5.5 µl lysis buffer containing streptavidin-coated donor beads/biotinylated cAMP tracer mix and 4.5 µl lysis buffer containing anti-cAMP conjugated acceptor beads and incubated for 2 h at RT in a dark place. The emission was measured at 520–620 nm on the EnVision™ reader (PerkinElmer). Concentration-response data from test compounds were analyzed with XLfit (IDBS) software using nonlinear regression applied to a sigmoidal dose-response model. cAMP concentration was derived from the cAMP standard curve, which was measured parallel to each assay.

2.3. Functional cAMP assay with transiently transfected COS7 cells

To assess the potency of LAAMA on calcitonin and amylin receptors of different species, cells were transiently transfected with the respective expression plasmids. The *in vitro* functional cAMP assay was used to measure accumulation of cAMP after receptor activation in the cells transiently transfected with pcDNA3.1 plasmids designed for expression of human, rat or mouse CTR alone or together with one of RAMPs. For transient transfection, COS7 cells were seeded on 384-well plates (7500 cells per well in volume of 50 µl culture medium). 25 µl of plasmid DNA/Attractene reagent (Qiagen) mix was added per well and the cells incubated for 4 h at 37 °C and 5% CO_2. The transfection medium was then replaced by culture medium (50 µl per well) and the cells incubated over night at 37 °C and 5% CO_2. Intracellular cAMP increase was measured using the AlphaScreen™ cAMP Functional Assay Kit (Perkin Elmer) as described above.

2.4. Animal husbandry and diet

Male RAMP 1/3 KO and WT mice were bred using the following breeding scheme: RAMP1/3 KO x RAMP1/3 KO and RAMP3 WT x RAMP3 WT (Coester et al., 2020), both genotypes were from the same generation F5N1 and were rederived at Janvier Elevage (Le Genest Saint Isle, France). Founder animals had been kindly provided by Prof. K. Caron, University of North Carolina, USA. Neuron specific CTR KO (Nestin-Cre^{CTR}) mice were bred at Janvier Elevage (Le Genest Saint Isle, France). To generate transgenic WT (Nestin-Cre^{WT}) and KO (Nestin-Cre^{CTR}) mice, male CTR floxed mice (Coester et al., 2020) (frozen sperm kindly provided by Drs. Jean-Pierre David and Thorsten Shinke, University Medical Center Hamburg) were crossed to female Nestin-Cre mice (JAX n°003771) generating CTR$^{fl/+}$: Nestin-Cre mice. These mice were again crossed with male CTR$^{fl/+}$ mice to generate the KO (Nestin-Cre^{CTR}) mice and the corresponding transgenic control mice (Nestin-Cre^{WT}). WT Nestin-WT^{WT} and floxed control mice Nestin-WT^{CTR} were also generated.

Mice were then transferred to the animal facility of the University of Zurich at the age of 6–12 weeks. All animals were group-housed at a constant temperature of approx. 22 °C, relative humidity of approx. 60%, and kept at a 12 h dark/light cycle with lights off at 10 h. Mice had free access to normal chow (65% carbohydrate, 22% protein, and 12.5% fat as percent of total energy content, cat. no. 3436; Provimi Kliba, Kaiseraugst, Switzerland) and tap water, and the cages were equipped with plastic housing and nesting material. After an acclimatization period, 7-week-old WT and RAMP1/3 KO and 7-13-week-old Nestin-Cre^{WT}, Nestin-Cre^{CTR}, Nestin-WT^{WT} and Nestin-WT^{CTR} mice were single housed to precisely monitor their food intake and were handled daily prior to the experiment to reduce stress related to substance administration. Two weeks before treatment, mice were placed on 45% high fat diet (HFD, 35% carbohydrate, 20% protein, and 45% fat as percent of total energy content, cat. no. D12451; Ssniff, Germany). One week before the treatment period, mice received daily sham subcutaneous injection of saline (NaCl 0.9%).

All animal experimental protocols in this study were approved by the University of Zurich Animal Protection Office and Ethics committee, the Veterinary Office of Canton Zurich (License n° 102/2018), and conform to Swiss Animal Protection guidelines and regulations (Swiss Animal Protection and Swiss Animal Act and Ordinance) and in accordance with the EU Directive on the protection of animals used for scientific purposes. These studies adhere to the ARRIVE guidelines.

2.5. Effect of LAAMA on body weight and food intake

After 2 weeks of HFD, single housed mice were injected subcutaneously daily for 7 days 1 h before dark onset. The RAMP 1/3 KO experiment consisted of four groups (n = 12–15/group): WT-Vehicle (D-Mannitol 200 mM) and WT-LAAMA (10 nmol/kg), and RAMP 1/3 KO-Vehicle and RAMP 1/3 KO-LAAMA (10 nmol/kg), respectively. The experiment with CTR KO mice also consisted of four groups (n = 19–20/group): Nestin-Cre^{WT} and Nestin-Cre^{CTR} were both injected with vehicle or LAAMA (10 nmol/kg). Food intake and body weight were measured daily and manually recorded.

To avoid possible degradation during long-term storage, LAAMA was freshly dissolved on each day of the study.

2.6. Mice killing and perfusion

Mice used to assess the effect of LAAMA on body weight and food intake were anesthetized with pentobarbital (100 mg/kg, i.p.; Kantonsapotheke Zurich, Switzerland) after 24 h from the last LAAMA injection. Blood was collected by terminal cardiac puncture into KEDTA tubes. Carcasses were stored at −20 °C until Echo-MRI scan analysis.

After a 12 h fast, to assess CTR immunostaining and LAAMA-induced c-Fos, separate cohorts from RAMP1/3 KO and Nestin-Cre^{CTR} mice colonies were injected i.p. with LAAMA (10 nmol/kg) or vehicle. 90 mins later, mice were anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused for 1.5 min with 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB-PFA, pH 7.4) for 2.5 min. Brains were isolated and postfixed overnight in 4% PB-PFA followed by overnight cryoprotection in 20% sucrose-0.1 M PB. They were then frozen in hexane on dry ice for 3 min and stored at −80 °C. Frozen hindbrains were cut at 20 μm in 3 series across the AP/NTS region and were mounted onto superfrost plus slides (Life Technologies Europe, Zug, Switzerland). Slides were stored in cryoprotectant (50% 0.02 M KPBS, 30% ethylene glycol, 20% glycerol) at −20 °C until staining for amylin-induced c-Fos and CTR fibers immunohistofluorescence (IHF) in the AP (Coester et al., 2020).

2.7. Echo-MRI analysis

Body composition of mice of all groups was measured post-mortem using an EchoMRI™ body composition analyzer (Zurich Integrative Rodent Physiology, UZH). Animals were placed in a plastic holder which was inserted in a tubular space in the EchoMRI™ system and body fat and lean mass were measured as a percent of body weight. Each measurement was performed in duplicate.

2.8. Immunohistofluorescence

2.8.1. c-Fos staining

Brain AP sections were washed in 0.02 M KPBS before being blocked for 2 h with 3% normal donkey serum (NDS), 0.3% Triton X-100 in 0.02 M KPBS at room temperature (RT). Single-label c-Fos IHF was carried out using rabbit anti-c-Fos antibody (1:500; 2250, Cell Signaling Technologies, BioConcept, Allschwil, Switzerland) and slides were incubated for 48 h at 4 °C. Slides were washed in 0.02 M KPBS 5 times for 5 min and incubated for 2 h with Cy3 donkey anti-rabbit antibody (1:100, 711-165-152, Jackson ImmunoResearch, LubioScience, Luzern, Switzerland). Sections were then counterstained with DAPI, and coverslipped using Vectashield Hardset mounting medium (Vectorlabs, Servion, Switzerland).

2.8.2. Calcitonin receptor staining

Brain AP sections were washed in 0.1% PBST (0.1% Triton X-100 in 0.01 M PBS) before being blocked for 90 min at RT with 3% NDS, 0.3% Triton X-100 in 0.01 M PBS. Slides were washed in 0.1% PBST and incubated for 48 h at 4 °C with rabbit anti-CTR antibody (1:1000, ab11042, Abcam, Cambridge, UK) in 0.3% PBST. Slides were washed twice in 0.01 M PBS followed by 10 min in 0.1% PBST. Secondary antibody (Alexa 647 donkey anti-rabbit 1:100 in 0.3% PBST, 711-605-152, Jackson ImmunoResearch) was then applied for 2 h at RT (Duffy et al., 2018; Potes et al., 2012). Finally, sections were washed in 0.01 M PBS, counterstained with DAPI, and coverslipped using Vectashield Hardset mounting medium.

2.9. Quantitative analysis of immunolabelled cells and fibers

C-Fos expressing neurons in the AP were imaged using an L2 Imager upright microscope (Zeiss, Germany). Images were set to the same threshold and c-Fos-positive neurons were quantified using ImageJ (NIH). Three AP sections were used for quantification.

For the quantitative analysis of CTR fiber density, three sections through the AP were acquired using a Zeiss SP8 confocal system equipped with a 20 × 0.75 objective (HD1 405, laser 29% with 471.3% gain, HyD3 laser 5% with 317% gain, laser zoom 1, pinhole 1, Z stack 19 μm, step of 1.5 μm). Slides were numerically coded to blind the experimenter. Image analysis was performed using ImageJ analysis software (NIH) as previously described (Lutz et al., 2018). Briefly, each image plane was set to a threshold and binarized. The integrated intensity, which reflects the total number of pixels in the binarized image, was then calculated. This procedure was conducted for each image plane in the stack, and the values were summed. The resulting value is an accurate index of the density of the nerve processes in the volume sampled. The same microscope setup (including objective, zoom, laser power, gain) was used to acquire all images within the same experiment. For representative images, images were equally adjusted for brightness and contrast within the same experiment.

2.10. Statistics

Statistical analysis was performed using unpaired *t*-test, 2-way ANOVA with repeated measure and 3-way ANOVA (factors: time, genotype and treatment), as appropriate, with multiple comparison post-hoc analysis using GraphPad prism 8 software.

3. Results

3.1. LAAMA is equipotent on human CTR and AMYs and its potency is conserved between human and rodent receptors

The pharmacological activity of LAAMA was evaluated in 1321N1 cells stably expressing human CTR, CGRPR (CLR + RAMP1) and ADMR (CLR + RAMP3) using a Gs mode cAMP assay (as a surrogate for pathway activation). Full concentration-responses (0.1 pM–10 μM) of LAAMA, human amylin, calcitonin (CT), CGRP and rat ADM were tested to compare the potency and selectivity of LAAMA to endogenous ligands. As shown in Fig. 1A and Table 1, LAAMA potently activated hCTR in a concentration-dependent manner. With the EC_{50} value of 1.5 nM, it was slightly less potent at the hCTR than hCT but approx. 4-fold more potent than hAmylin (Fig. 1A, Table 1 and Suppl. Table 1). LAAMA did not activate hCGRPR and hADMR in the cAMP assay at any concentration tested (Fig. 1B and C and Table 1), suggesting high selectivity for CTR. *In vitro* functional cAMP assay with transiently transfected COS7 cells showed that LAAMA was equipotent on all three AMYs (Fig. 1D–G) and LAAMA compound action on CTR was preserved between human, rat and mouse (Fig. 1G–I).

3.2. LAAMA is a long acting peptide

LAAMA is a peptide stabilized by lipidation, which facilitates binding to serum albumin to prevent a fast excretion by kidney. The PK of the LAAMA in plasma was investigated in lean rats following i.v. and s.c. administration. The terminal half-life in lean rats was 34 h, as desired for a long-acting peptide. A maximum concentration of 78.7 nM after a single s.c. administration of 20 nmol/kg was reached at 24 h post dose. PK in mouse has been assessed for similar peptides from the same peptide class with the identical half-life extension as the here described LAAMA and were comparable to rat PK results.

3.3. Effect of repeated administration of LAAMA on food intake and body weight in WT and RAMP1/3 KO mice

To test whether body weight and food intake lowering effects of LAAMA require RAMP1/3, WT and RAMP1/3 KO mice kept on HFD were once-daily s.c. injected with vehicle or 10 nmol/kg LAAMA over the period of 7 days. Body weight (Suppl. Fig 1A) was calculated as cumulative body weight gain (Fig. 2A) or as a percent of baseline (Suppl.

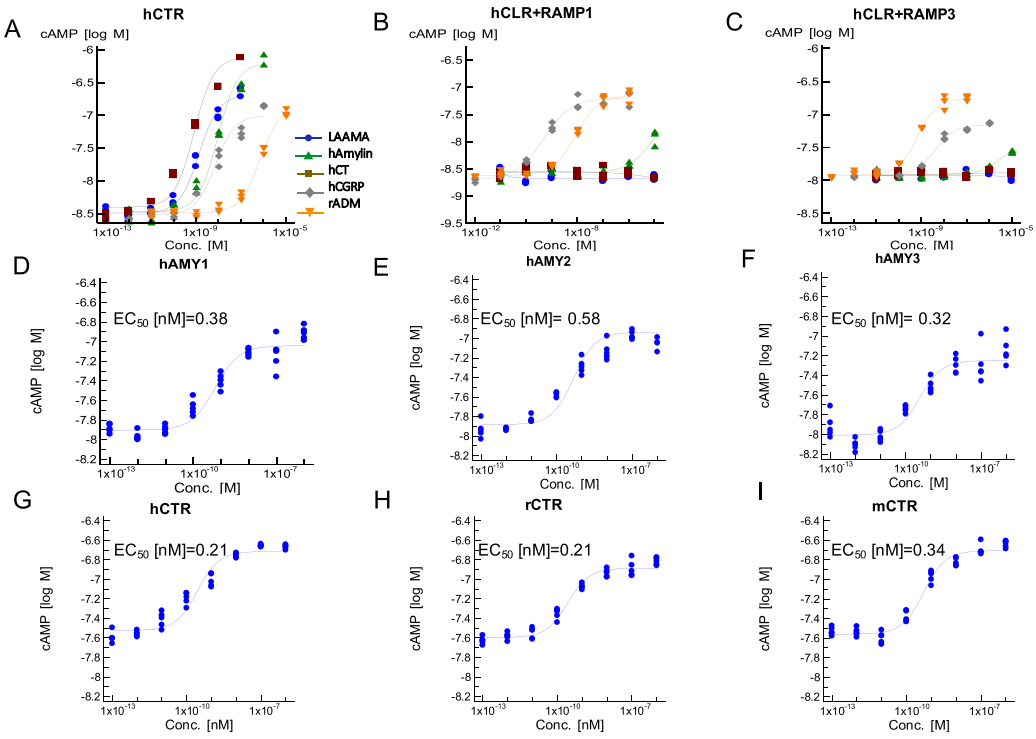

Fig. 1. LAAMA is equipotent on human CTR/CALCR and AMYs and its action is conserved between human and rodent receptors. (A–C) Representative concentration-response curves showing cAMP accumulation in cells stably expressing (**A**) hCTR, (**B**) hCLR + Ramp1 (**C**) hCLR + Ramp3 after activation with LAAMA, hAmylin, hCalcitonin, hCGRP and rAdrenomedullin. (D–I) Representative concentration-response curves showing cAMP accumulation in cells transfected with (**D**) hAMY1, (**E**) hAMY2, (**F**) hAMY3, (**G**) hCTR, (**H**) rat CTR, (**I**) mouse CTR after activation with LAAMA. Abbr. hCTR -human calcitonin receptor, RAMP-receptor activity modifying protein, cAMP- cyclic adenosine monophosphate, hCGRP- human calcitonin gene-related peptide, hAMY-human amylin receptor, EC_{50}-half maximal effective concentration, value is the geometric mean of three independent experiments.

Table 1
Activity of LAAMA, human amylin, human calcitonin (hCT), hCGRP and rat adrenomedullin (rAMD) on human calcitonin receptor (hCTR), hCGRPR, hAMDR. (relates to Fig. 1). EC_{50} is calculated as the geometric mean of three independent experiments.

	LAAM EC_{50} [nM]	hAmylin EC_{50} [nM]	hCT EC_{50} [nM]	hCGRP EC_{50} [nM]	rADM EC_{50} [nM]
hCTR	1.5	6.1	0.43	11	1295
hCGRPR (hCLR + RAMP1)	>10,000	>10,000	>10,000	0.54	12
hAMDR (hCLR + RAMP3)	>10,000	>10,000	>10,000	4.1	0.95

Fig 1B). Repeated treatment with LAAMA significantly decreased body weight in WT and RAMP 1/3 KO mice compared to their respective controls. (Fig. 2A and Suppl. Fig. 1A and B). Daily food intake was similar among the four groups, though a slight tendency towards reduced food intake was observed in the LAAMA treated when compared to vehicle treated groups, however without being statistically significant (Fig. 2B and Suppl. Fig.1C). There was no effect of LAAMA on terminal glucose levels in WT or KO mice (Fig. 2C). However, glucose was higher in WT-Vehicle group compared to RAMP 1/3 KO-Vehicle ($P = 0.0017$) and to RAMP 1/3 KO-LAAMA mice ($P = 0.0004$; Fig. 2C). WT-LAAMA mice also showed higher glucose levels compared to RAMP1/3 KO-LAAMA mice ($P = 0.0041$; Fig. 2C). Body composition analysis did not reveal any statistically significant difference among the groups (Fig. 2D and E).

3.4. LAAMA induces a c-Fos response in the area postrema of WT mice only

To test whether LAAMA activates neurons in the AP (the brain region known to express CTR and RAMPs and to mediate the eating-inhibitory effect of endogenous amylin (Lutz et al., 2001; Mollet et al., 2004)) and whether this effect requires intact RAMP1/3, the c-Fos response was measured in WT and RAMP1/3 KO mice peripherally treated with vehicle or 10 nmol/kg LAAMA. The overall cell count was low, but LAAMA induced a 9-fold increase in the c-Fos response in the AP of WT mice compared to vehicle controls ($P = 0.029$; Fig. 3A and B). C-Fos response was also increased in the AP of RAMP1/3 KO mice (Fig. 3A and B) compared to their vehicle injected counterpart, however, this effect was statistically not significant.

3.5. Effect of repeated administration of LAAMA on food intake and body weight in Nestin-Cre^{WT} and Nestin-Cre^{CTR} mice

To test whether the effects of LAAMA on food intake and body weight are mediated by neuronal CTR, we next depleted CTR from Nestin positive neurons.

Among the 4 mouse groups, Nestin-WT^{WT} and Nestin-WT^{CTR} displayed higher body weight than Nestin-Cre^{CTR} and Nestin-Cre^{WT} suggesting that the cre phenotype is significantly different than the WT phenotype (Suppl. Fig. 2A–C). Thus, the Nestin-Cre^{WT} mice were chosen as controls for the following experiments.

The neuronal CTR-KO (Nestin-Cre^{CTR}) mice and corresponding control (Nestin-Cre^{WT}) mice were kept on HFD and peripherally treated with 10 nmol/kg LAAMA (once-daily, over a period of 7 days). Treatment with LAAMA significantly reduced body weight gain of Nestin-Cre^{WT} mice compared to their respective vehicle-treated group (Fig. 4A, Supp. Fig. 3A and B). Body weight gain of Nestin-Cre^{CTR} mice was also

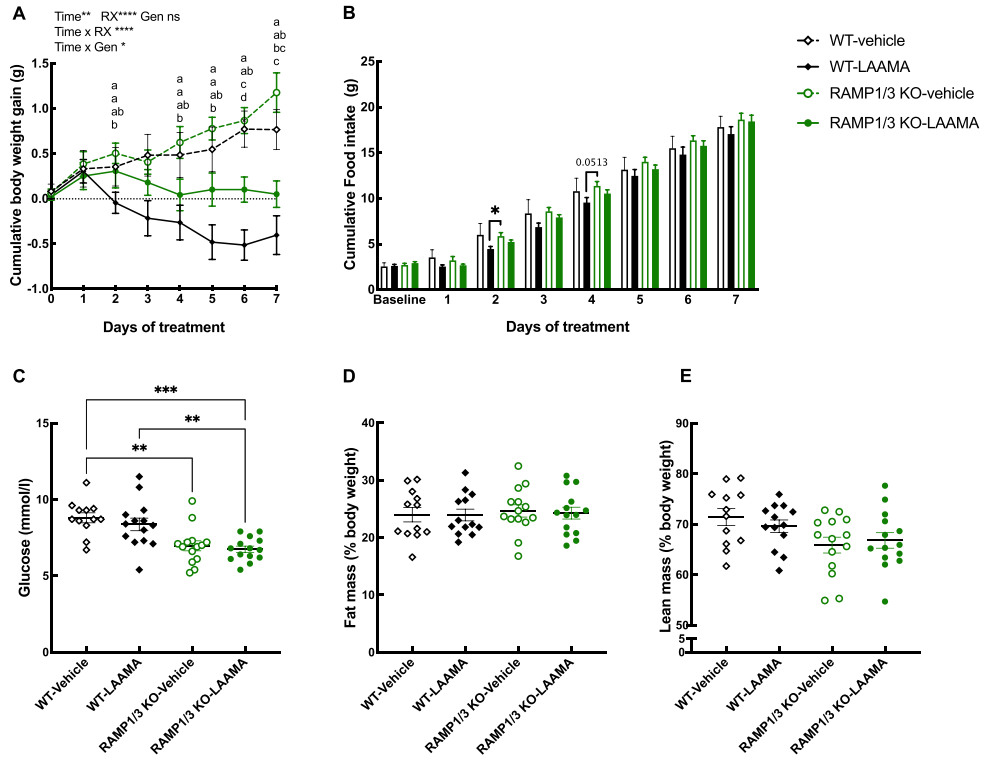

Fig. 2. Effect of LAAMA on food intake and body weight in WT and RAMP1/3 KO mice compared to vehicle.
(**A**) Cumulative body weight gain over the 7 day-period; (**B**) Cumulative food intake over the 7 day-period; (**C**) Glucose at sacrifice in mmol/L; (**D**) Fat mass at sacrifice expressed as a percent of final body weight; (**E**) Lean mass at sacrifice expressed as a percent of final body weight. Data are presented as mean ± S.E.M., N = 12–15/group. Statistics: Data are analyzed with a 3-way and 2-way ANOVA (factors: genotype, treatment (RX) and time) followed by Tukey's multiple comparisons test. Parameters with differing letters (a, b, c, d) differ from each other by at least $P < 0.05$. *P < 0.05; **P < 0.01, ***P < 0.001, ns: not significant.

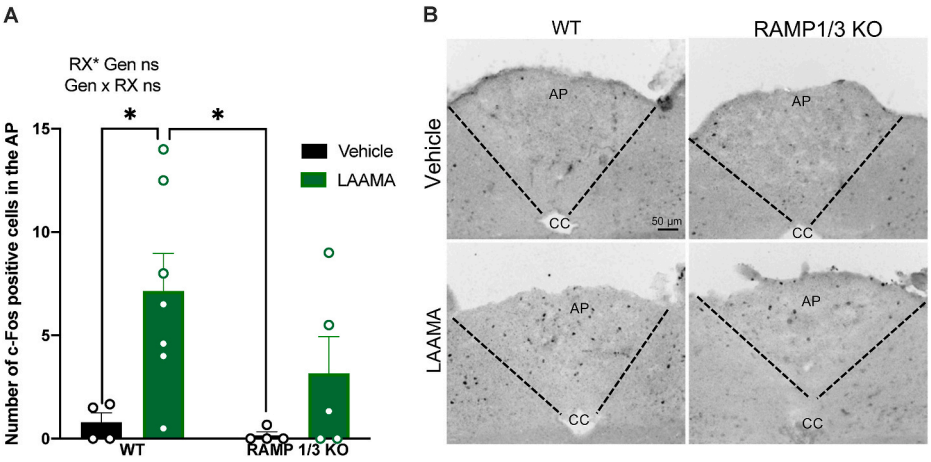

Fig. 3. LAAMA induces a c-Fos response in the area postrema (AP) of WT mice only.
(**A**) Quantification of c-Fos positive neurons; (**B**) Representative images of AP brain sections immunostained for c-Fos in WT vs. RAMP1/3 KO male mice 90 min after vehicle or LAAMA (10 nmol/kg) injection (20× magnification). Values are presented as mean ± S.E.M. n = 4–7/group. Statistics: two-way ANOVA (mixed effect analysis) followed by Tukey's multiple comparisons test and unpaired T-test. *P < 0.05. ns: not significant.

reduced when treated with LAAMA compared to their respective vehicle group, however this effect was significantly weaker than for Nestin-Cre^{WT} mice (Fig. 4A, Supp. Fig. 3A and B). In line with the effects on body weight, cumulative food intake was reduced in LAAMA-treated Nestin-Cre^{WT} animals compared to the respective non-treated group. On the contrary, LAAMA did not reduce daily food intake in Nestin-Cre^{CTR} mice (Fig. 4B, Suppl. Fig.3C).

3.6. LAAMA does not induce a c-Fos response in Nestin-Cre^{CTR} mice compared to Nestin-WT^{CTR}

To confirm the mouse model, CTR fiber immunodensity was quantified in the AP of vehicle injected mice, with Nestin-Cre^{CTR} mice showing an 86% decrease compared to Nestin-Cre^{WT} group ($P < 0.05$; Fig. 4C and D).

To test whether the activation of AP neurons by LAAMA requires intact CTR, Nestin-Cre^{CTR} and Nestin-WT^{CTR} mice were peripherally injected with LAAMA (10 nmol/kg). Treatment with LAAMA induced an 18-fold increase of c-Fos in Nestin-WT^{CTR} compared to their vehicle-treated counterpart ($p = 0.0144$; Suppl. Fig. 4A and B). In contrast, no c-Fos induction was observed in Nestin-Cre^{CTR} mice (Suppl. Fig. 4A and B).

4. Discussion

In order to investigate the role of the different AMY components in conveying the effects of LAAMA on eating and body weight, we determined LAAMA's *in vitro* potency on CTR, AMYs, CGRPR and ADMR and *in vivo* efficacy in RAMP 1/3 KO and Nestin-Cre^{CTR} mice. First, we showed that LAAMA equipotently activated CTR and AMYs *in vitro*, while being inactive on other tested receptors. Moreover, potency of LAAMA is preserved between human, rat and mouse CTRs, making it a

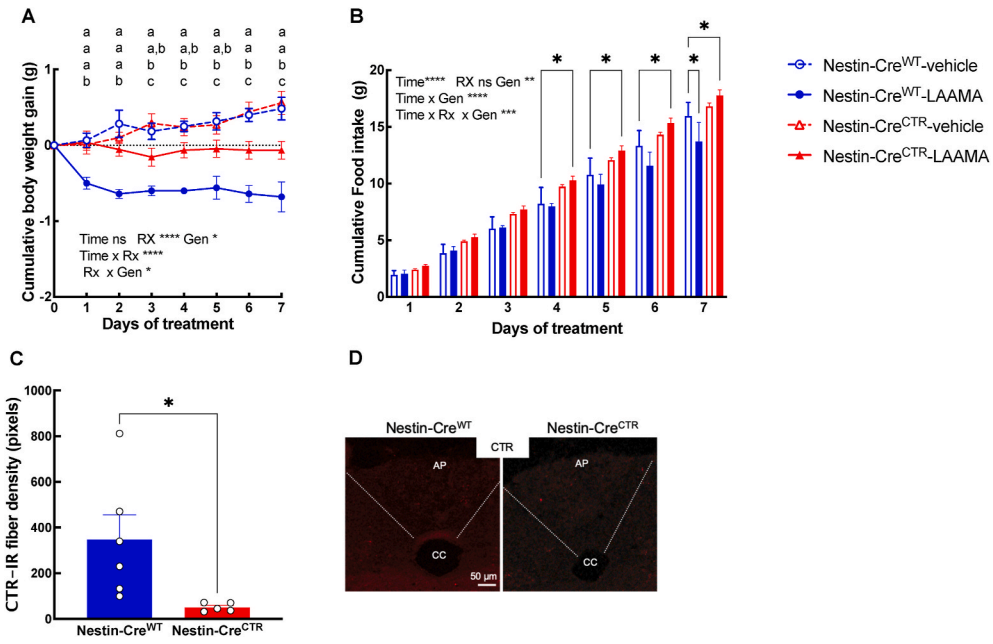

Fig. 4. CTR immunodensity quantification and effect of LAAMA on food intake and body weight in Nestin-Cre^{WT} and Nestin-Cre^{CTR} mice compared to vehicle. (**A**) Cumulative body weight gain over the 7 day-period; (**B**) Cumulative food intake over the 7 day-period; (**C**) Quantification of CTR fiber immunodensity in the AP; (**D**) Representative images of CTR fiber immunostaining in the AP of Nestin-Cre^{CTR} and Nestin-Cre^{CTR} male mice (20× magnification). Data are presented as mean ± S.E.M. N = 5–11/group. Statistics: Data are analyzed with a 3-way ANOVA (factors: genotype, treatment (RX) and time) followed by Tukey's multiple comparisons test. Parameters with differing letters (a,b,c) differ from each other by at least $P < 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

suitable tool for *in vivo* studies in both rodent species. *In vivo*, LAAMA decreased body weight gain in WT as well as in RAMP 1/3 KO mice, indicating that RAMP1/3 are not crucial for LAAMA-induced effects. In contrast, absence of neuronal CTR (Nestin-Cre^{CTR}) resulted in less pronounced reduction of body weight after treatment with LAAMA, suggesting that neuronal CTR is the main mediator of LAAMA's body weight lowering effects.

In vitro studies showed that LAAMA equipotently increased intracellular cAMP, an established marker of AMYs and CTR activation (Armour et al., 1999; Gingell et al., 2014; Perry et al., 1997; Qi et al., 2013; Udawela et al., 2006), after binding to CTR or AMYs. This suggests that unlike amylin, LAAMA's efficacy to activate downstream signaling pathways in CTR expressing cells is not altered by RAMPs. It is important to consider that even though the increase of intracellular cAMP after LAAMA or amylin treatment means that the receptor has been activated *in vitro* (Bower and Hay, 2016), it is not always accompanied by an anorectic effect *in vivo* (Hay et al., 2015). C-Fos, on the other hand, has been traditionally shown as a marker of neuronal activation *in vivo* after amylin treatment (Barth et al., 2004; Christopoulos et al., 1995; Riediger et al., 2004; Sexton et al., 1994). Our *in vivo* studies showed that LAAMA increased c-Fos by 9-folds and 18-folds in the AP of WT and transgenic Nestin-Cre control mice, respectively, compared to vehicle-treated animals. RAMP 1/3 KO mice had a partial, although not statistically significant c-Fos response to LAAMA, but notably, the overall number of c-Fos positive neurons was low. We have previously shown that RAMP 1/3 KO mice display a strong decrease in CTR density in the AP (Coester et al., 2020), which may have contributed to the lower number of LAAMA induced c-Fos positive cells as we have also seen with amylin (Coester et al., 2020). This assumption agrees with the results obtained from the Nestin-Cre^{CTR} mice. In these animals, injection of LAAMA had no measurable c-Fos response. Our results of c-Fos induction *in vivo* confirmed *in vitro* data indicating that CTR expressing cells with or without RAMPs can mediate LAAMA's effects. In line with this conclusion, LAAMA reduced body weight gain to the same extent in both, WT and RAMP1/3 KO mice. Unchanged food intake suggests that LAAMA may induce body weight loss through increased energy expenditure, but further studies are needed to confirm this hypothesis.

The importance of neuronal CTR for the effects of LAAMA on body weight was confirmed by the results obtained from the Nestin-Cre^{CTR} mice. These animals were less responsive to the repeated LAAMA administration than the transgenic controls (Nestin-Cre^{WT}). CTR fiber density staining in the AP of Nestin-Cre^{CTR} and the transgenic control mice confirmed the absence of neuronal CTR.

5. Conclusion

Our research advanced the understanding of LAAMA's actions *in vivo* and *in vitro*. We demonstrated that the novel drug LAAMA binds CTR and AMYs equipotently *in vitro* and induces intracellular cAMP synthesis. *In vivo,* LAAMA significantly reduced body weight in WT and in RAMP 1/3 KO mice, while animals with the neuronal CTR KO were less responsive showing that unlike amylin, LAAMA does not necessarily require RAMPs to reduce body weight and that LAAMA effects are mainly mediated by the neuronal CTR.

CRediT authorship contribution statement

Salome Gamakharia: Methodology, Investigation, Writing – original draft, Writing – review & editing, guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. **Christelle Le Foll:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. **Wolfgang Rist:** Investigation. **Tamara Baader-Pagler:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Resources. **Angela Baljuls:** Conceptualization, Methodology, Writing – review & editing, Resources. **Thomas A. Lutz:** Conceptualization, Writing – review & editing, Resources, Funding acquisition, has received research support from investigator-initiated sponsored proposals from Boehringer-Ingelheim Pharma.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: W.R., A.B. and T.B.P. are full-time employees of Boehringer-Ingelheim Pharma.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174352.

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